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Origin of the *Diplazium hachijoense* complex (Athyriaceae)

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Abstract

We analyzed the phylogeny of the *Diplazium hachijoense* complex using plastid *trnL-F* and low-copy nuclear marker *AK1* DNA sequences. Based on allele constitution, triploid apogamous species of the *D. hachijoense* complex appeared to have originated from the hybridization of triploid apogamous species and diploid sexual species by recurrent hybridization events. These results suggested that triploid apogamous ferns can achieve hybridization with diploid sexual species by producing diploid spores with irregular meiosis in sporogenesis. Furthermore, the present study predicted the involvement of several unknown species associated with hybridization. More sampling of *Callipteris* species from China and adjacent areas is required to determine the relationships among unknown species and the *D. hachijoense* complex.

Keywords

apogamous, Athyriaceae, Ferns, *Diplazium*, hybridization, phylogeny

Introduction

In sexually reproductive fern species, meiosis produces 64 haploid spores per sporangium following mitotic divisions of spore mother cells four times, and each mother cell contains half of the parental chromosome number (Manton 1950). By contrast, apogamous ferns produce 32 spores per sporangium by one of two pathways to yield chromosomally unreduced diplospores (Grusz 2016): premeiotic endomitosis (Döpp 1939, Manton 1950) or meiotic first division restitution (Braithwaite 1964). Most apogamous ferns produce their spores by the former pathway (Manton 1950). In ad-

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dition, a few apogamous species possess a third mechanism of reproduction that yields either 16 or 64 relatively regular shaped spores per sporangium (Park and Kato 2003).

Apogamous reproduction is not an unusual feature in ferns. Approximately 3% of all fern species (Liu et al. 2012) and 13% of Japanese fern species, for which information regarding their reproductive modes is available, reportedly exhibit apogamous reproduction (Takamiya 1996). Although several apogamous fern species do not require sexual reproduction throughout their life cycles, they exhibit extensive morphological and genetic variation and often form species complexes with continuous morphological variation. Numerous studies have reported reticulate relationships between apogamous and sexual fern species (e.g., Watano and Iwatsuki 1988, Suzuki and Iwatsuki 1990, Lin et al. 1995, Grusz et al. 2009, Chao et al. 2012, Dyer et al. 2012, Ebihara et al. 2012, Hori et al. 2014), which are exhibited in four patterns (Figure 1): (1) tetraploid hybrids are formed between triploid apogamous species and diploid sexual species, (2) triploid hybrids are formed between diploid apogamous species and diploid sexual species, (3) triploid hybrids are formed between triploid apogamous species and diploid sexual species , and (4) tetraploid hybrids are formed between triploid apogamous species and tetraploid sexual species. In patterns (1) and (2), diploid or triploid apogamous species generate unreduced sperm (Walker 1962, Watano and Iwatsuki 1988, Grusz et al. 2009, Chao et al. 2012, Jaruwattanaphan et al. 2013, Dyer et al. 2012) or eggs (Dyer et al. 2012, Hori et al. 2014, Hori et al. 2018a) that are united with a reduced gamete from a sexual species. Alternatively, in patterns (3) and (4), a reduced gamete from a sexual species is united with a reduced diploid sperm or egg generated by an apogamous species (Ebihara et al. 2012, Hori et al. 2014, Hori et al. 2018b, Hori 2018c).

Lin et al. (1992) proposed the hybridization cycle hypothesis as the mechanism of recurrent hybridization that occurs in triploid apogamous species after the discovery of triploid apogamous *Dryopteris pacifica*, which produces diploid spores through irregular meiosis. If prothallia of such diploid spores can produce eggs or sperm, an apogamous hybrid species can repeatedly originate from the hybridization of apogamous and sexual species without an increase in ploidy. This hypothesis is supported by numerous reports of irregular meiosis in spore mother cells of triploid Polypodiales apogamous species: *Athyrium* (Athyriaceae, Kurita 1964; Hirabayashi 1970, Park and Kato 2003), *Deparia* (Athyriaceae, Hirabayashi 1970), *Diplazium* (Athyriaceae, Takamiya et al. 1999), *Cyrtomium* (Dryopteridaceae, Hirabayashi 1970), and *Dryopteris* (Dryopteridaceae, Hirabayashi 1967, Lin et al. 1992).

Diplazium hachijoense Nakai (Athyriaceae) is one of the most common triploid apogamous ferns in Japan (Takamiya et al. 1999, Ebihara 2017). It is difficult to identify because of the continuous morphological variation between other closely related triploid apogamous species (*D. conterminum*, *D. dilatatum*, *D. doederleinii*, *D. okinawaense*, *D. taiwanense*, *D. takii*, and *D. virescens*), diploid sexual species (*D. amamianum*), and tetraploid sexual species (*D. nipponicum*). In addition, *D. dilatatum* has a sexual diploid cytotype (Takamiya et al. 1999), and *D. doederleinii* has an apogamous

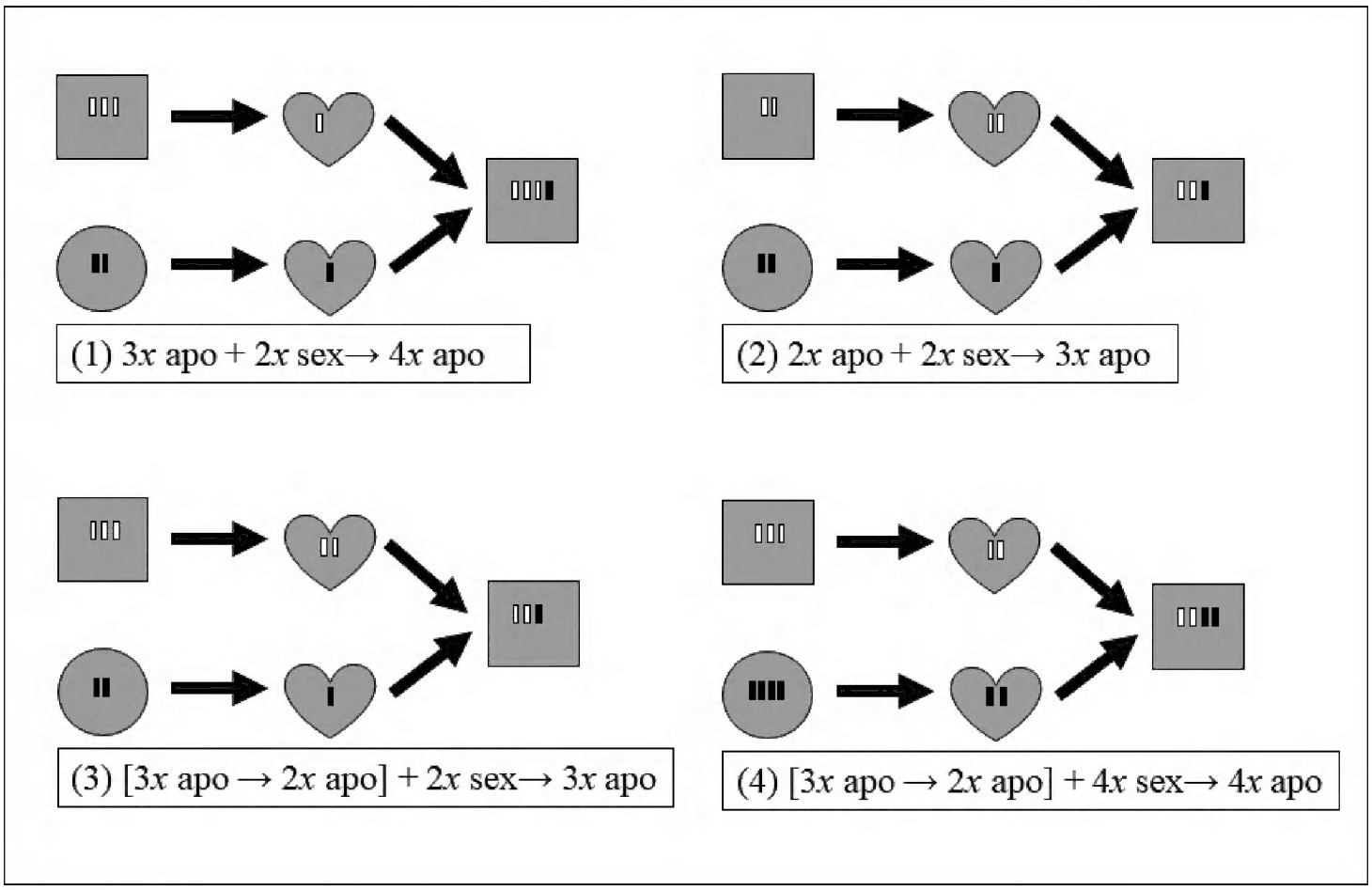

Figure 1. Hybridization patterns with germination from which apogamous species are derived. (**1**) Tetraploid hybrid between a triploid apogamous species and a diploid sexual species, (**2**) triploid hybrid between a diploid apogamous species and a diploid sexual species, (**3**) triploid hybrid between a triploid apogamous species and a diploid sexual species, (**4**) tetraploid hybrid between a triploid apogamous species and a tetraploid sexual species. Circle, sporophyte of sexual species; heart, gametophyte; square, sporophyte of apogamous species.

Table 1. The ploidy level and reproductive mode of the *D. hachijoense* complex in previous studies.

Species	Reproducive mode	ploidy level	Refereces
D. conterminum	apogamous	3×	Takamiya et al. 1999
D. dilatatum	apogamous	3×	Takamiya et al. 1999
D. doederleinii	apogamous	3×	Takamiya et al. 1999
D. doederleinii	apogamous	4×	Takamiya et al. 2001
D. okinawaense	apogamous	3×	Takamiya et al. 1999
D. taiwanense	apogamous	3×	Takamiya et al. 1999
D. takii	apogamous	3×	Takamiya et al. 1999
D. virescens	apogamous	3×	Takamiya et al. 1999
D. amamianum	sexual	2×	Takamiya et al. 1999
D. nipponicum	sexual	4×	Takamiya et al. 1999

tetraploid cytotype (Takamiya et al. 2001). The ploidy level and reproductive mode of the *D. hachijoense* complex in previous studies are both shown in Table 1. The present study aimed to determine the hybridization patterns between apogamous and sexual species within the *D. hachijoense* complex.

Materials and methods

Plant materials

In this study, all 10 species of the *Diplazium hachijoense* complex and an additional four species, which have not yet been assigned scientific names and termed as *Diplazium* sp. 1–4, were investigated. *Diplazium chinense*, *D. esculentum*, *D. fauriei*, *D. mettenianum*, *Deparia japonica*, *De. viridifrons*, *De. unifurcata*, *Athyrium crenulatoserrulatum*, and *A. decurrentialatum* were used as outgroups. Voucher information for all samples is listed in Appendix 1. All voucher specimens have been deposited in the Makino Herbarium of Tokyo Metropolitan University, Aichi University of Education, and/or the Kochi Prefectural Makino Botanical Garden.

Chromosome count and assessment of reproductive modes

We counted mitotic chromosomes from *D. amamianum*, *D. dilatatum*, *D. hachijoense*, *D. nipponicum*, *D. takii*, and *Diplazium* sp. 2–4 (localities are listed in Appendix 1). Chromosome counting methodologies were performed as outlined in Takamiya et al. (1999). To assess the reproductive modes of each sample or herbarium specimen, the number of spores/sporangium were counted. Specimens were considered sexual if the number of spores/sporangium was 64; they were considered apogamous if the number of spores/sporangium was 32 (Manton 1950).

DNA extraction

For molecular analyses, total DNA was extracted from silica-dried leaves using cetyltrimethylammonium bromide solution, according to Doyle and Doyle (1990).

Plastid and nuclear DNA sequencing

trnL-F was used as the maternally-inherited plastid DNA marker (F: 5'-ATTTGAACTGGTGACACGAG-3' and FernL 1 Ir1: 5'-GGYAATCCTGAGCAAATC-3'; Taberlet et al. 1991, Li et al. 2009). *AK1* (AK4F: 5'-GATGAAGCCATCAAGAAACCA-3' and AKR2: 5'-ATGGATCCAGCGACCAGTAA-3'; Hori et al. 2018b) was used as a biparentally-inherited nuclear marker for polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) analysis, which was used to determine allelic variation in each individual (Ebihara et al. 2012, Jaruwattanaphan et al. 2013).

PCR amplification was performed using PrimeSTAR Max DNA Polymerase (Takara, Kyoto, Japan). PCR entailed an initial denaturation step at 95 °C for 10 min,

followed by 35 cycles of denaturation, annealing, and elongation steps at 98 °C for 10 s, 55 °C for 5 s, and 72 °C for 5 s, respectively, using a Model 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA).

Gel electrophoresis of *AK1* PCR products was performed using gels containing 2% glycerol at 15 °C for 16 h at 300 V, followed by silver staining. For sequencing of the bands separated on the SSCP gels, the polyacrylamide gel was dried after silver staining by sandwiching the gel between Kent paper and a cellophane sheet on an acrylic back plate at 55 °C for 3 h. To extract the DNA, a piece of the DNA band was peeled from the dried gel using a cutter knife and incubated in 50 μL of Tris-EDTA buffer (10-mM Tris-HCl and 1-mM EDTA, pH 8.0) at 25 °C overnight. The supernatant solution was used as a template for further PCR amplification with the same primer set employed for initial PCR amplification.

PCR products were purified using ExoSAP-IT (USB, Ohio, USA) or Illustra ExoStar 1-Step (GE Healthcare, Wisconsin, USA) and used as templates for direct sequencing. Reaction mixtures for sequencing were prepared using the BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems). The reaction mixtures were analyzed using an ABI 3130 Genetic Analyzer (Applied Biosystems). All plant samples were classified based on their PCR-SSCP banding patterns, and each band was DNA sequenced.

Molecular analysis

For phylogenetic analyses, the sequences were typified and made non-redundant by removing duplicate sequences. Only one sequence representing each allele for *AK1* and for each haplotype for *trnL-F* were used in the datasets (Appendices 1, 2). The sequences were aligned using MUSCLE (Edgar 2004) and assessed with Bayesian inference (BI) analysis using MrBayes 3.2.6 (Ronquist et al. 2012) and maximum parsimony (MP) analysis using the MEGA X software (Kumar et al. 2018). In the BI analysis, the best-fit model of sequence evolution for each DNA region was selected using jModelTest 2.1.10 (Darriba et al. 2012; *trnL-F*: HKY+G model; *AK1*: HKY model). In addition, we assessed BI (*trnL-F*: HKY+I+G model; *AK1*: HKY model) and MP analysis with full-data sets. Four Markov chain Monte Carlo chains were run simultaneously and sampled every 100 generations for 1 million generations in total. Tracer 1.7.1 (Rambaut et al. 2018) was used to examine the posterior distribution of all parameters and their associated statistics, including estimated sample sizes. The first 2,500 sample trees from each run were discarded as burn-in periods. The MP tree was obtained using the subtree pruning-regrafting algorithm (Swofford et al. 1996) at search level 1, at which the initial trees were obtained by the random addition of sequences (10 replicates). Indels were treated as missing characters in the MP and BI analyses. The confidence level of the monophyletic groups was estimated with 1,000 MP bootstrap pseudo-replicates.

Results

Chromosome count and estimation of reproductive mode

The ploidy level and reproductive mode of *D. hachijoense* complex species was consistent with previous reports (Takamiya et al. 1999): *D. amamianum* and *D. dilatatum*, $2n = 82$, diploid sexual; *D. hachijoense* and *D. takii*, $2n = 123$, triploid apogamous; and *D. nipponicum*, $2n = 164$, tetraploid sexual (Appendix 1).

Plastid and nuclear DNA phylogenetic trees

We sequenced 719–748 bp of the *trnL-F* intergenic spacer from different specimens. The aligned *trnL-F* matrix was 748 bp, of which 114 characters (15%) were parsimony-informative. For the *AK1* intron, we sequenced 280–520 bp of the intron for each specimen, yielding a 574 bp aligned matrix, of which 74 characters (13%) were parsimony-informative. The MP trees derived from our *trnL-F* and *AK1* sequence analyses with BI posterior-probabilities (PP) and MP bootstrap percentages (BP) are shown in Figures 2a, 3a, respectively. In the phylogenies with full-data set of *trnL-F* and *AK1* (Figures 2b, 3b, respectively), 118 characters (15%) and 84 characters (14%) were parsimony-informative, respectively.

To define allelic types of the *D. hachijoense* complex, we investigated which diploid sexual or autotriploid apogamous species had each allele supported by PP and BP (Figures 2a–3b and Table 2). Alleles of nuclear genes from samples whose sequences formed a clade with particular lineages were considered to originate from the parental species. Therefore, when two alleles from one triploid apogamous species formed clades with those of parental species A and B, the allele composition of the apogamous species was AB. Unfortunately, PCR-SSCP analysis could not distinguish among the genotypes A1A1B1, A1B1B1, and A1B1 as this method cannot determine the quantity of each allele in PCR products. Therefore, the present study only showed the alleles obtained from each material, not their proportions, in Table 2 and Appendix 1.

To divide each allele number with the alphabet, we used clades supported by BP, PP, and similarity in the sequences. Furthermore, we investigated which diploid sexual or autotriploid apogamous species had each allele (Table 2). Based on allelic relationships shown in Figures 2a–3b and Table 2, *D. dilatatum* seemed to have only type A, *D. takii* had only B, and *D. doederleinii* had only C for both *trnL-F* and *AK1* sequences. Type D containing *D. amamianum* was more clearly monophyletic because the BP and PP values were higher than those for *D. dilatatum*, *D. takii*, and *D. doederleinii*. Regarding other alleles of undetected (or missing) species, we could not conclude which alleles came from the same species. Therefore, we tentatively treated these alleles as individually distinct species, outlining them as E, F, G, H, J and K.

In total, five types of plastid *trnL-F* haplotypes (Type α–ε) and 10 types of nuclear *AK1* alleles (Type A–H, J, and K) were recovered from the *D. hachijoense* complex

Table 2. Reproductive mode, ploidy level, plastid haplpotype (*trnL-F* intergenetic spacer), and nuclear allele (*AK1*) of the *D. hachijoense* complex in this study. Rep, reproductive mode; sex., sexual; apo., apogamous; ploi., ploidy level. Any allelic types of nuclear gene *AK1* that were identified by sequencing are in boldface.

Voucher	Locality	Species	rep.	ploi.	*trnL-F*	*AK1*
S. Serizawa 91648-1	Okinawa pref.: Kunigami village, Mt. Yonahadake	*D. dilatatum*	apo.		α1	**A3 and A4**
S. Serizawa 91648-2	Okinawa pref.: Nago city, Genka	*D. dilatatum*			α1	**A1A4**
K. Hatake 705	Kagoshima pref.: Amami city, Sumiyou village, Santaro-touge, 350m alt.	*D. dilatatum*	sex.	2×	α2	**A1**
K. Hatake 974	Kagoshima pref.: Amami city, Naze	*D. dilatatum*	sex.	2×	α1	**A4**
K. Hori 3082	Kagoshima pref.: Yakushima Is, Koseda, 70m alt.	*D. dilatatum*			α1	**A6**
K. Hori 3083	Kagoshima pref.: Yakushima Is, Koseda, 70m alt.	*D. dilatatum*			α1	**A1A2**
K. Hori 3125	Kagoshima pref.: Yakushima Is, Hara, 80m alt.	*D. dilatatum*			α1	**A6**
M. Takamiya 866	Mie pref.: Minamimuro county, Kiho-cho	*D. takii*	apo.	3×	β1	**B2**
K. Hori 2924	Fukuoka pref.: Kasuya county, Hisayama-machi, 140m alt.	*D. takii*			β1	**B2**
K. Hori 2958	Fukuoka pref.: Kasuya county, Hisayama-machi, 140m alt.	*D. takii*			β1	**B2**
K. Hori 2343	Mie pref.: Minamimuro county, Kiho-cho, 70m alt.	*D. takii*			β1	**B2B3**
K. Hori 3173	Kagoshima pref.: Yakushima Is, Isso-river, 390m alt.	*D. doederleinii*			γ2	**C**
K. Hatake 615	Kasgoshima pref.: Amami city, Naze, Honchya-touge, 250m alt.	*D. amamianum*	sex.	2×	δ1	D1
K. Hatake 985	Kasgoshima pref.: Amami city, Sumiyou village, Santaro-touge, 350m alt.	*D. amamianum*	sex.	2×	δ1	**D1**
K. Hatake 609	Kasgoshima pref.: Amami city, Naze, Ooaza-asato	*D. amamianum*	sex.	2×	δ4	**D1**
K. Hori 3084	Kagoshima pref.: Yakushima Is, Koseda, 70m alt.	*D. taiwanense*			β2	**A2B1**
K. Hori 3080	Kagoshima pref.: Yakushima Is, Koseda, 71m alt.	*D. taiwanense*	apo.		α1	**A1A6B1**
K. Hori 3087	Kagoshima pref.: Yakushima Is, Isso-river, 200m alt.	*D. okinawaense*	apo.		δ2	**A1B2**
S. Serizawa 91663-1	Okinawa pref.: Nago city, Genka	*D.* sp. 1	apo.		α1	**A1A5G**
K. Hori 3158	Kagoshima pref: Yakushima Is, Tabugawa, 200m alt.	*D. conterminum*			β1	**B2F**
K. Hori 2341	Mie pref.: Minamimuro county, Kiho-cho	*D. virescens*			β1	**B2E**
K. Hori 2342	Mie pref.: Minamimuro county, Kiho-cho	*D. virescens*			β1	**B2E**
K. Hori 3086	Kagoshima pref.: Yakushima Is, Miyanoura river, 20m alt.	*D. virescens*			β1	**B1B2E**
K. Hatake 773	Kagoshima pref.: Tokunoshima Is, Mt. Inokawadake, 200m alt.	*D. hachijoense*	apo.	3×	δ1	**B2D1**
K. Hatake 776	Kagoshima pref.: Tokunoshima Is, Mt. Inokawadake, 200m alt.	*D. hachijoense*	apo.	3×	δ2	B2D1
K. Hori 1681	Chiba pref.: Katori county, Tako-machi, Hayashi	*D. hachijoense*			δ3	**B2D2**
S. Serizawa 91664-1	Okinawa pref.: Nago city, Genka	*D. hachijoense*	apo.		δ1	B2D2
K. Hori 2957	Fukuoka pref.: Kasuya county, Hisayama-machi, 140m alt.	*D. hachijoense*			δ3	B2D2
M. Takamiya 528	Yamaguchi pref.: Nagato city, Ichinoo	*D. hachijoense*			δ3	B2D2
M. Takamiya 919	Mie pref.: Minamimuro county, Kiho-cho	*D. hachijoense*	apo.	3×	δ5	**B2D3**
K. Hatake 1010	Shizuoka pref.: Shimoda city, Renndaiji-onsenn, 100m alt.	*D. hachijoense*	apo.	3×	δ5	B2D3
K. Hatake 395	Kagoshima pref.: Tokunoshima Is, Mt. Inokawadake, 200m alt.	*D. hachijoense*	apo.	3×	δ1	**B2D1**
M. Takamiya 883	Mie pref.: Minamimuro county, Kiho-cho	*D. hachijoense*	apo.	3×	δ3	**B2D2**
M. Takamiya 893	Mie pref.: Minamimuro county, Kiho-cho	*D. hachijoense*	apo.	3×	δ3	**B2D2**
M. Takamiya 1883	Kagoshima pref.: Kagoshima city, Chuzann-cho, Takinoshita-river	*D.* sp. 3	apo.	3×	δ1	**CD2**
S. Serizawa 91654-1	Okinawa pref.: Kunigami village, Mt. Yonahadake	*D.* sp. 2			γ1	**CK**
S. Serizawa 91654-2	Okinawa pref.: Kunigami village, Mt. Yonahadake	*D.* sp. 2			γ1	CK
K. Hori 2338	Mie pref.: Minamimuro county, Kiho-cho	*D.* sp. 4			δ3	**D1H1**
M. Takamiya 929	Mie pref.: Minamimuro county, Kiho-cho	*D.* sp. 4	apo.	3×	δ1	D2H2
K. Hatake 1030	Kumamoto pref.: Amakusa city	*D.* sp. 4	apo.	3×	δ1	**D2H2**
K. Hori 2339	Mie pref.: Minamimuro county, Kiho-cho	*D. nipponicum*			ε	**D2J**
K. Hatake 1004	Kanagawa pref.: Minamiashigara city, Kano	*D. nipponicum*	sex.	4×	ε	**D2J**

a

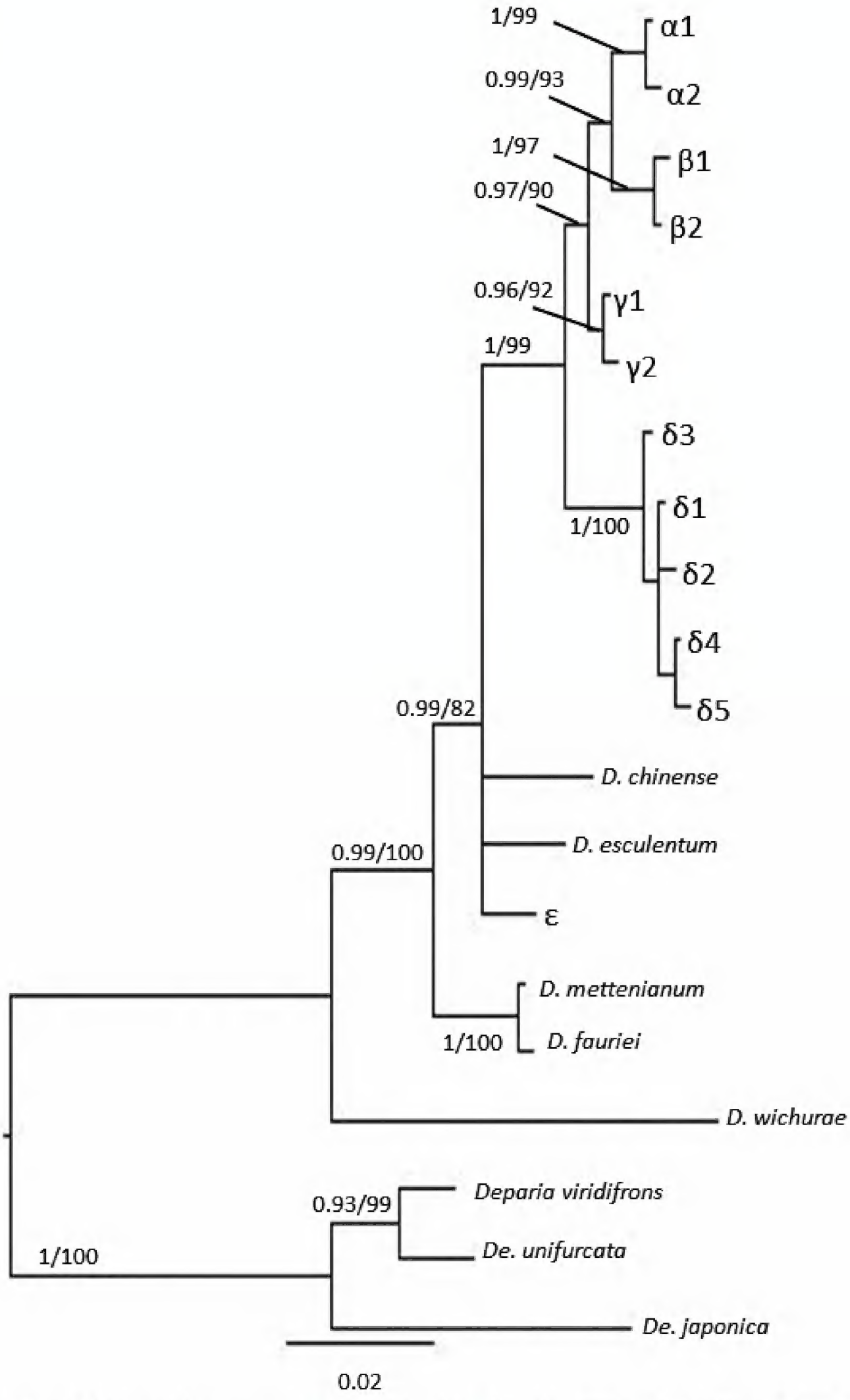

Figure 2a. A 50% majority consensus tree resulting from Bayesian Markov chain Monte Carlo Bayesian (B/MCMC) analysis of plastid intergenic spacer *trnL-F* with BI PP (>0.95) and MP BP (>70) node support values. The sequences were typified and made non-redundant by removing duplicate sequences.

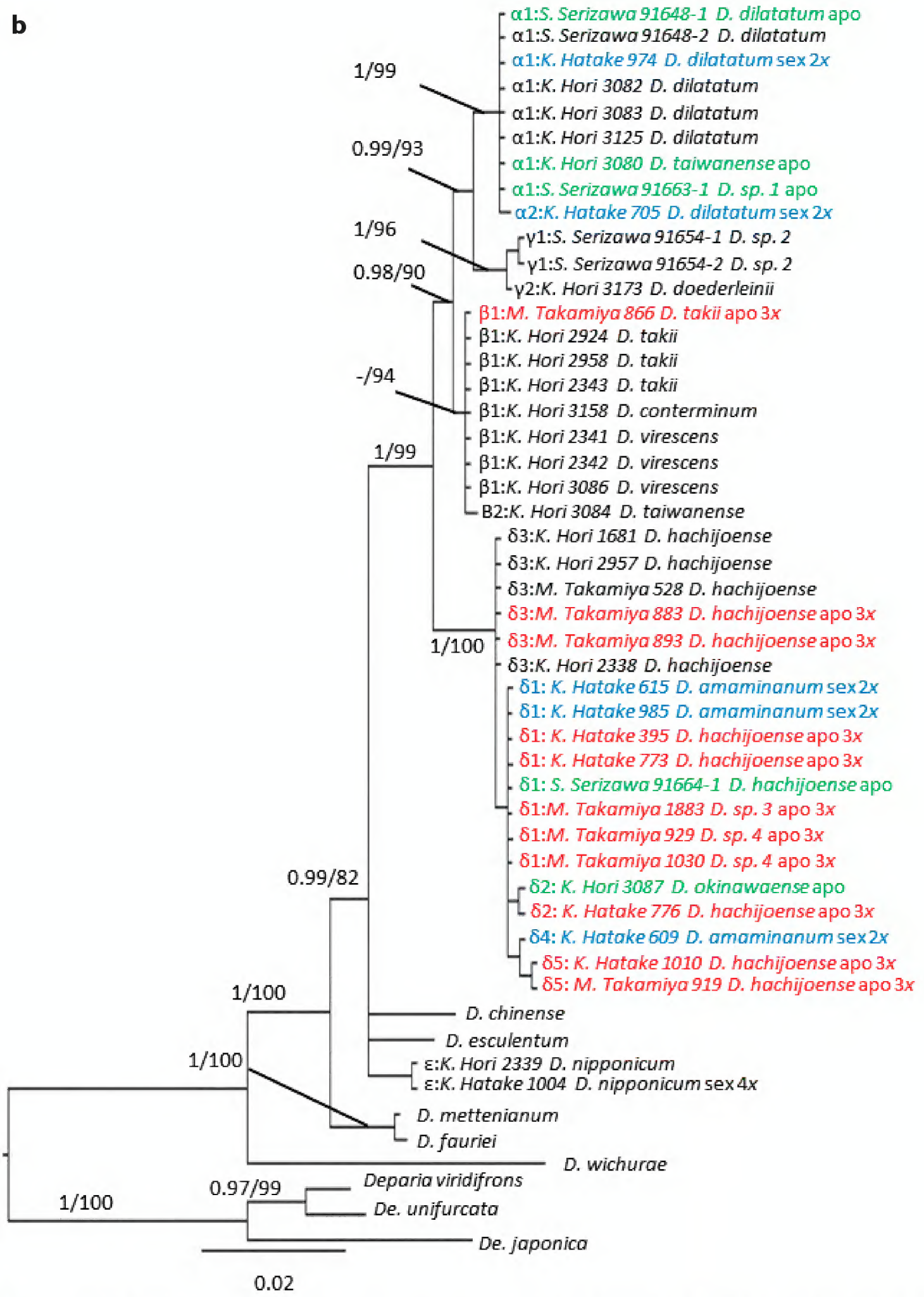
b
α1:S. Serizawa 91648-1 D. dilatatum apo
α1:S. Serizawa 91648-2 D. dilatatum
α1:K. Hatake 974 D. dilatatum sex 2x
α1:K. Hori 3082 D. dilatatum
α1:K. Hori 3083 D. dilatatum
α1:K. Hori 3125 D. dilatatum
α1:K. Hori 3080 D. taiwanense apo
α1:S. Serizawa 91663-1 D. sp. 1 apo
α2:K. Hatake 705 D. dilatatum sex 2x
γ1:S. Serizawa 91654-1 D. sp. 2
γ1:S. Serizawa 91654-2 D. sp. 2
γ2:K. Hori 3173 D. doederleinii
β1:M. Takamiya 866 D. takii apo 3x
β1:K. Hori 2924 D. takii
β1:K. Hori 2958 D. takii
β1:K. Hori 2343 D. takii
β1:K. Hori 3158 D. conterminum
β1:K. Hori 2341 D. virescens
β1:K. Hori 2342 D. virescens
β1:K. Hori 3086 D. virescens
B2:K. Hori 3084 D. taiwanense
δ3:K. Hori 1681 D. hachijoense
δ3:K. Hori 2957 D. hachijoense
δ3:M. Takamiya 528 D. hachijoense
δ3:M. Takamiya 883 D. hachijoense apo 3x
δ3:M. Takamiya 893 D. hachijoense apo 3x
δ3:K. Hori 2338 D. hachijoense
δ1: K. Hatake 615 D. amaminanum sex 2x
δ1: K. Hatake 985 D. amaminanum sex 2x
δ1: K. Hatake 395 D. hachijoense apo 3x
δ1: K. Hatake 773 D. hachijoense apo 3x
δ1: S. Serizawa 91664-1 D. hachijoense apo
δ1:M. Takamiya 1883 D. sp. 3 apo 3x
δ1:M. Takamiya 929 D. sp. 4 apo 3x
δ1:M. Takamiya 1030 D. sp. 4 apo 3x
δ2: K. Hori 3087 D. okinawaense apo
δ2: K. Hatake 776 D. hachijoense apo 3x
δ4: K. Hatake 609 D. amaminanum sex 2x
δ5: K. Hatake 1010 D. hachijoense apo 3x
δ5: M. Takamiya 919 D. hachijoense apo 3x
D. chinense
D. esculentum
ε:K. Hori 2339 D. nipponicum
ε:K. Hatake 1004 D. nipponicum sex 4x
D. mettenianum
D. fauriei
D. wichurae
Deparia viridifrons
De. unifurcata
De. japonica
0.02
1/99
0.99/93
1/96
0.98/90
-/94
1/99
1/100
0.99/82
1/100
1/100
0.97/99
1/100

Figure 2b. Full-data set of a 50% majority consensus tree resulting from Bayesian Markov chain Monte Carlo Baysean (B/MCMC) analysis of plastid intergenic spacer *trnL-F* with BI PP (>0.95) and MP BP (>70) node support values. Blue, diploid sexual; red, triploid apogamous; green, apogamous but ploidy was not estimated in this study.

a

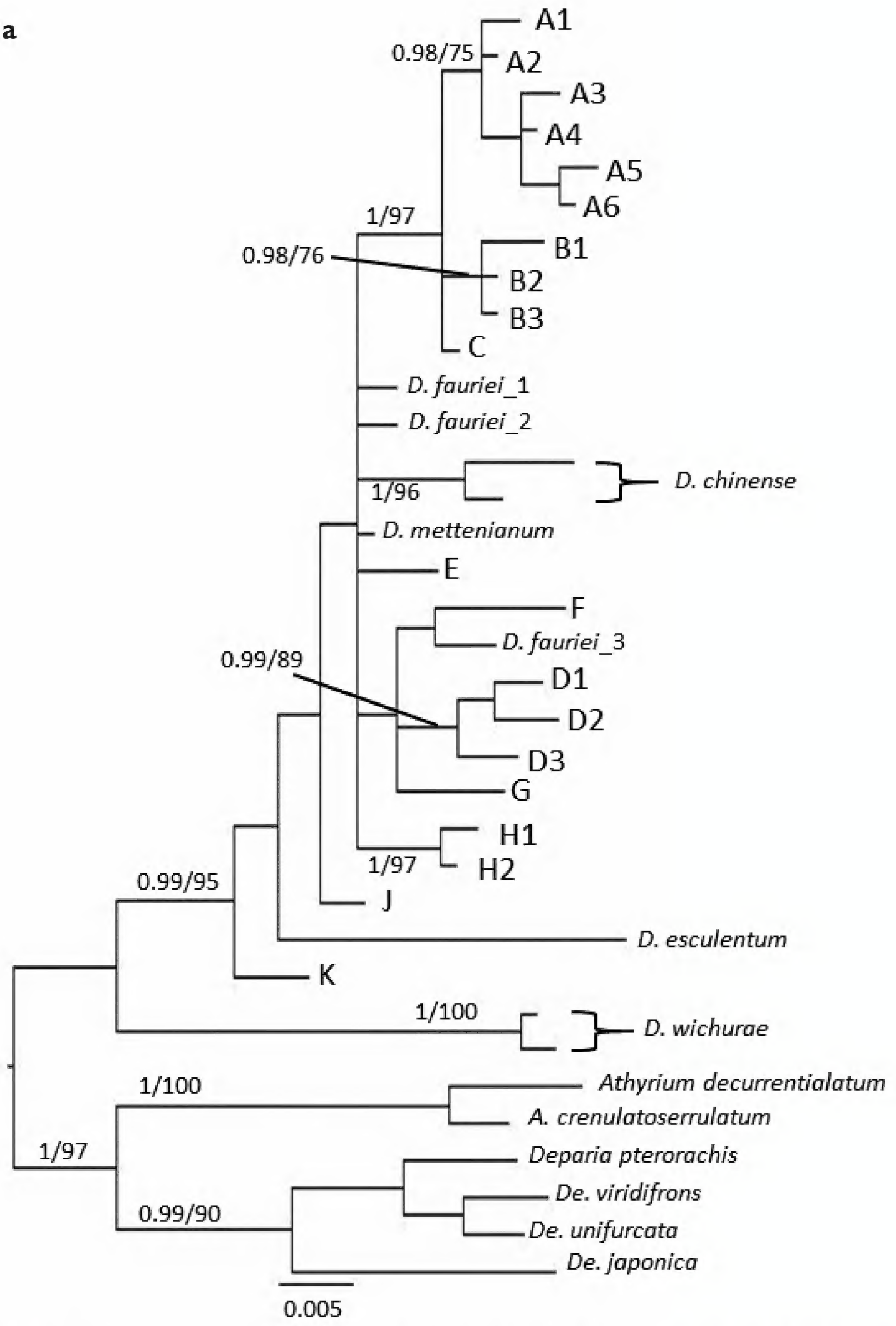

Figure 3a. A 50% majority consensus tree resulting from Bayesian Markov chain Monte Carlo Bayesian (B/MCMC) analysis of the nuclear gene *AK1* with BI PP (>0.95) and MP BP (>70) node support values. The sequences were typified and made non-redundant by removing duplicate sequences.

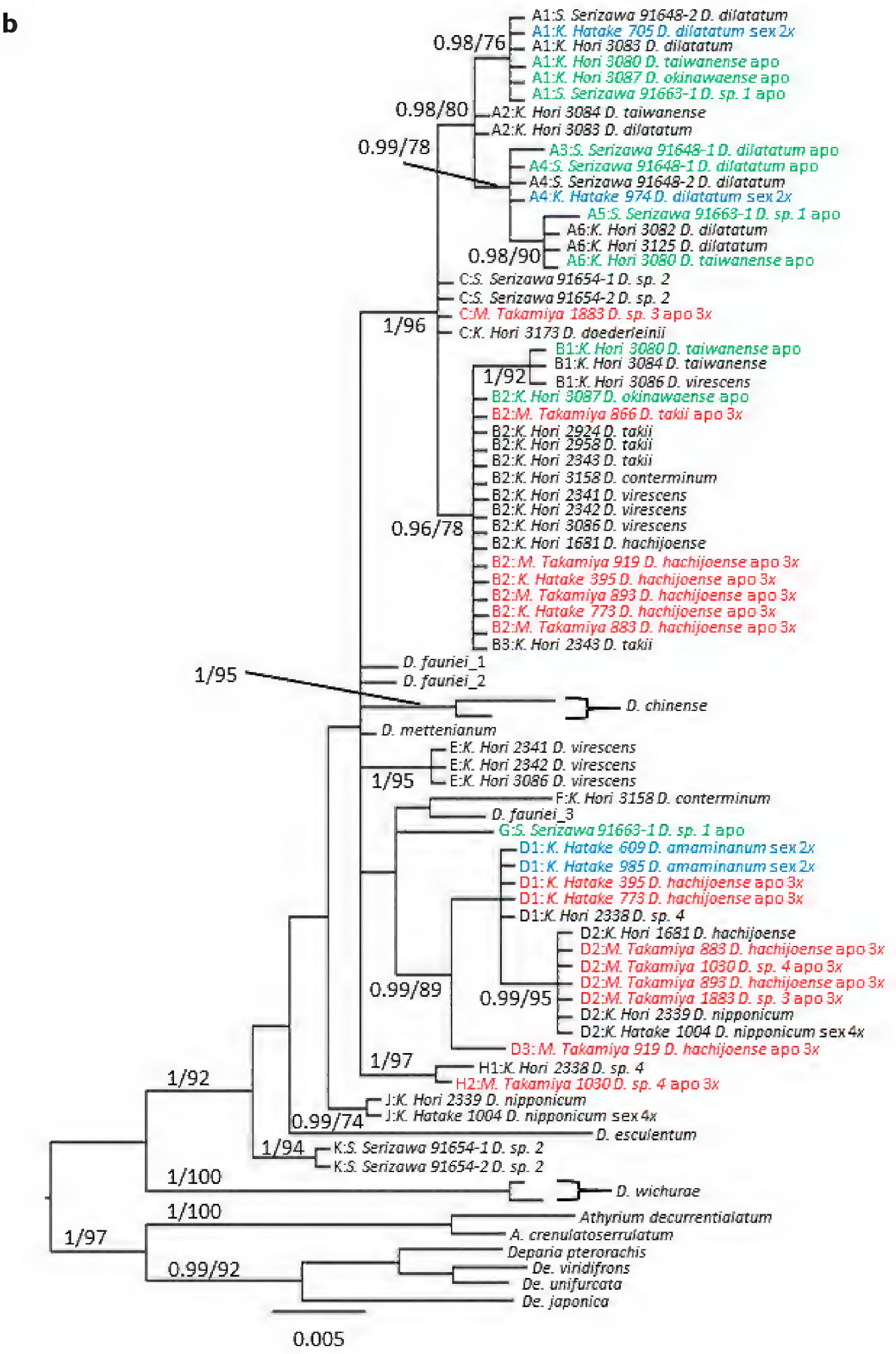
b
A1:S. Serizawa 91648-2 D. dilatatum
A1:K. Hatake 705 D. dilatatum sex 2x
0.98/76
A1:K. Hori 3083 D. dilatatum
A1:K. Hori 3080 D. taiwanense apo
A1:K. Hori 3087 D. okinawaense apo
A1:S. Serizawa 91663-1 D. sp. 1 apo
0.98/80
A2:K. Hori 3084 D. taiwanense
A2:K. Hori 3083 D. dilatatum
0.99/78
A3:S. Serizawa 91648-1 D. dilatatum apo
A4:S. Serizawa 91648-1 D. dilatatum apo
A4:S. Serizawa 91648-2 D. dilatatum
A4:K. Hatake 974 D. dilatatum sex 2x
A5:S. Serizawa 91663-1 D. sp. 1 apo
A6:K. Hori 3082 D. dilatatum
A6:K. Hori 3125 D. dilatatum
0.98/90
A6:K. Hori 3080 D. taiwanense apo
C:S. Serizawa 91654-1 D. sp. 2
C:S. Serizawa 91654-2 D. sp. 2
C:M. Takamiya 1883 D. sp. 3 apo 3x
1/96
C:K. Hori 3173 D. doederleinii
B1:K. Hori 3080 D. taiwanense apo
B1:K. Hori 3084 D. taiwanense
1/92
B1:K. Hori 3086 D. virescens
B2:K. Hori 3087 D. okinawaense apo
B2:M. Takamiya 866 D. takii apo 3x
B2:K. Hori 2924 D. takii
B2:K. Hori 2958 D. takii
B2:K. Hori 2343 D. takii
B2:K. Hori 3158 D. conterminum
B2:K. Hori 2341 D. virescens
B2:K. Hori 2342 D. virescens
0.96/78
B2:K. Hori 3086 D. virescens
B2:K. Hori 1681 D. hachijoense
B2:M. Takamiya 919 D. hachijoense apo 3x
B2:K. Hatake 395 D. hachijoense apo 3x
B2:M. Takamiya 893 D. hachijoense apo 3x
B2:K. Hatake 773 D. hachijoense apo 3x
B2:M. Takamiya 883 D. hachijoense apo 3x
B3:K. Hori 2343 D. takii
D. fauriei_1
1/95
D. fauriei_2
D. chinense
D. mettenianum
E:K. Hori 2341 D. virescens
E:K. Hori 2342 D. virescens
1/95
E:K. Hori 3086 D. virescens
F:K. Hori 3158 D. conterminum
D. fauriei_3
G:S. Serizawa 91663-1 D. sp. 1 apo
D1:K. Hatake 609 D. amaminanum sex 2x
D1:K. Hatake 985 D. amaminanum sex 2x
D1:K. Hatake 395 D. hachijoense apo 3x
D1:K. Hatake 773 D. hachijoense apo 3x
D1:K. Hori 2338 D. sp. 4
D2:K. Hori 1681 D. hachijoense
D2:M. Takamiya 883 D. hachijoense apo 3x
D2:M. Takamiya 1030 D. sp. 4 apo 3x
0.99/89
D2:M. Takamiya 893 D. hachijoense apo 3x
0.99/95
D2:M. Takamiya 1883 D. sp. 3 apo 3x
D2:K. Hori 2339 D. nipponicum
D2:K. Hatake 1004 D. nipponicum sex 4x
D3:M. Takamiya 919 D. hachijoense apo 3x
1/97
H1:K. Hori 2338 D. sp. 4
H2:M. Takamiya 1030 D. sp. 4 apo 3x
1/92
J:K. Hori 2339 D. nipponicum
0.99/74
J:K. Hatake 1004 D. nipponicum sex 4x
D. esculentum
1/94
K:S. Serizawa 91654-1 D. sp. 2
K:S. Serizawa 91654-2 D. sp. 2
1/100
D. wichurae
1/100
Athyrium decurrentialatum
1/97
A. crenulatoserrulatum
Deparia pterorachis
0.99/92
De. viridifrons
De. unifurcata
De. japonica
0.005

Figure 3b. Full-data set of a 50% majority consensus tree resulting from Bayesian Markov chain Monte Carlo Baysean (B/MCMC) analysis of the nuclear gene *AK1* with BI PP (>0.95) and MP BP (>70) node support values. Blue, diploid sexual; red, triploid apogamous; green, apogamous but ploidy was not estimated in this study.

(Table 2). Plastid haplotypes in the *D. hachijoense* complex were as follows (Fig. 2a, Table 2): sexual or apogamous, type α- *D. dilatatum*, *D. taiwanense*, and *Diplazium* sp. 1; type β- *D. conterminum*, *D. taiwanense*, *D. takii*, and *D. virescens*; type γ- *D. doederleinii* and *Diplazium* sp. 2; type δ- *D. amamianum*, *D. hachijoense*, *D. okinawaense*, *Diplazium* sp. 3, and *Diplazium* sp. 4; and type ε- *D. nipponicum*. Types α, β, γ, and δ were well supported by PP (>0.95) and BP (>90) values. In the phylogeny with full-data set, Type ε was also supported, but Type γ was not supported by PP.

Allelic constitution of *AK1* in the *D. hachijoense* complex were as follows (Fig. 3a, Table 2): sexual or apogamous, type A- *D. dilatatum*; type B- *D. takii*; type C- *D. doederleinii*; type D- *D. amamianum*; *D. taiwanense*, one or two allele A and one allele B; *D. hachijoense*, one allele B and D; *D. okinawaense*, one allele A and B; *D. nipponicum*, one allele D and J; *D.* sp. 1, two allele A and one allele G; *D.* sp. 2, C and K; *D.* sp. 3, C and D; and *D.* sp. 4, D and H. Types A, B, D, and H were rather supported by PP (>0.95) and BP (>70) values. In the phylogeny with full-data set, Type E, J, and K were also supported.

Discussion

Figure 4 represents the reticulogram of the *D. hachijoense* complex. The ploidy levels and reproductive modes of undetected species are unknown; thus, we assigned them as either diploid sexual species (E, F, G, J, and K) or triploid apogamous species (H). If undetected species H was a diploid sexual species, we could not explain the origin of triploid apogamous *Diplazium* sp. 4 (nuclear *AK1*=D and H) because allele D belongs to diploid sexual *D. amamianum*. Thus, it must be a diploid hybrid.

The allelic constitution in *D. hachijoense* suggested that it resulted from hybridization between the diploid sexual species *D. amamianum* and the triploid apogamous species *D. takii*. Because plastid genomes are reported to be maternally-inherited in ferns (Gastony and Yatskievych 1992), *D. amamianum* is likely the maternal parent of *D. hachijoense*. *trnL-F* sequences of *D. amamianum* and *D. hachijoense* are united in Clade δ (Figure 2). The two *AK1* alleles present in *D. hachijoense* are united in Clades B and D, with alleles from *D. takii* and *D. amamianum*, respectively. Furthermore, *D. taiwanense* and *Diplazium* sp. 3 exhibited allelic constitutions to similar to that of *D. hachijoense*, suggesting closely allied progenitors. *Diplazium taiwanense* comprises both A and B *AK1* biparentally-inherited alleles. However, one specimen of *D. taiwanense* possessed an α maternally-inherited plastid haplotype derived from *D. dilatatum*, whereas the other specimen of *D. taiwanense* possessed a β plastid haplotype. The former species had one A and B allele, while the latter had two A alleles and one B allele in *AK1*. This suggested recurrent, reciprocal origins of *D. taiwanense*. In the first hybridization event, apogamous *D. takii* is the maternal parent of *D. taiwanense*, whereas sexual *D. dilatatum* is the maternal parent of *D. taiwanense* in the second hybridization event. *Diplazium* sp. 3 has C and D alleles of *AK1* derived from *D. doederleinii* and *D. amamianum*, respectively, and the δ plastid haplotype derived from *D. amamianum*.

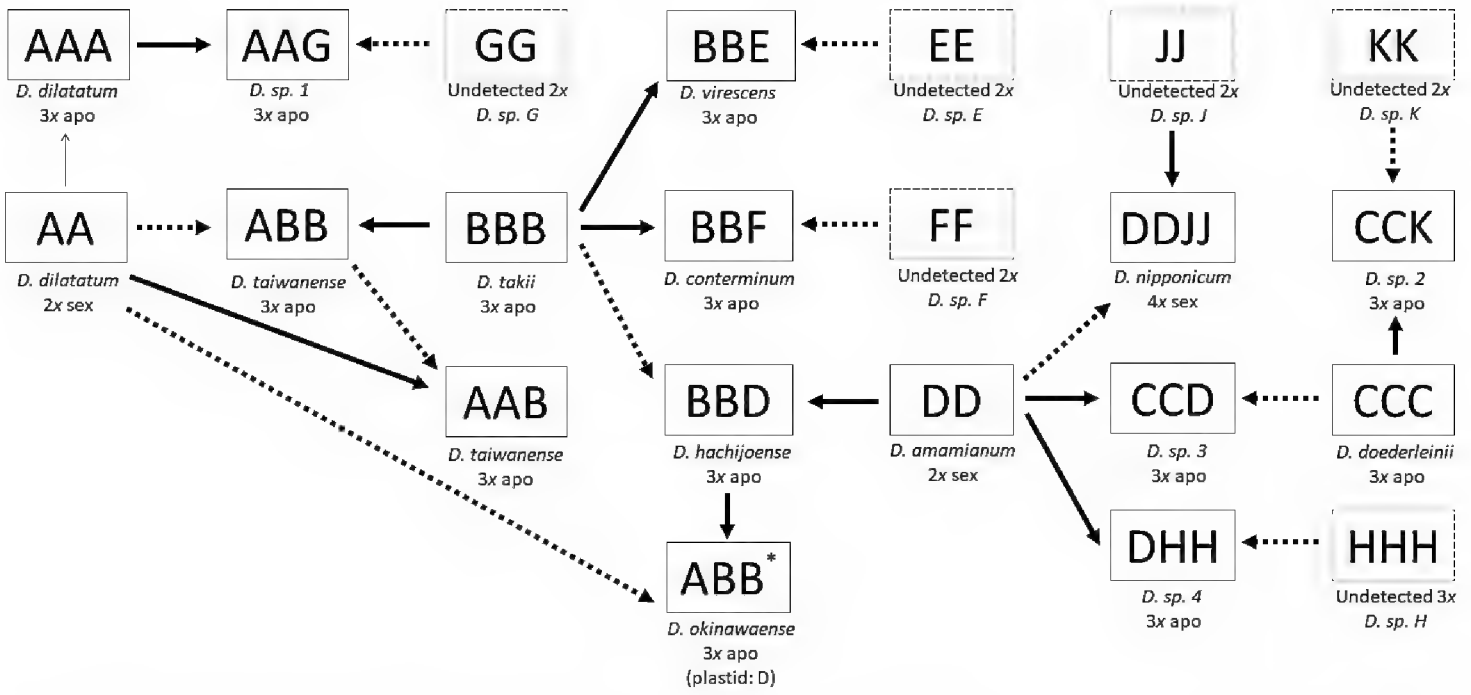

Figure 4. The reticulogram of the *D. hachijoense* complex. Thin-solid arrow, autopolyploidization; Solid arrows, maternal inheritance; dashed arrows, paternal inheritance; square, triploid apogamous or diploid sexual species; dashed square, hypothesized diploid sexual or triploid apogamous species. **D. okinawaense* had only nuclear *AK1* allele A of *D. dilatatum* and B of *D. takii*, although plastid haplotype was D of *D. amaminanum*.

However, *Diplazium* sp. 3 was difficult to distinguish from *D. hachijoense* because of similar morphological characteristics. Further investigations are required to determine whether *Diplazium* sp. 3 is a novel species.

The allelic constitution of *D. okinawaense* also suggested recurrent hybridization. The *trnL-F* phylogeny suggested that *D. amamianum* is the maternal progenitor of *D. okinawaense*. However, the *AK1* allelic constitution of *D. okinawaense* is A and B, neither of which is found in *D. amamianum*. The inconsistency between the plastid haplotype and the nuclear allelic constitution of *D. okinawaense* may reflect recurrent hybridization events between the triploid apogamous species *D. hachijoense* as the maternal parent and the sexual diploid *D. dilatatum*. Such a scenario may have resulted in the loss of *D. amamianum* nuclear alleles through genetic segregation with recombination (Figure 4).

Allelic constitution in other species suggested that there were six undetected parental species which have only one allele E, F, G, J, K, or H. This study could not resolve ploidy and reproductive mode of these species. Tentatively, in the reticulogram (Figure 4), we proposed *Diplazium* sp. E, F, G, J, and K as hypothesized diploid sexual species and *Diplazium* sp. H as triploid apogamous species. In the reticulogram, we interpreted the origin of six apogamous and one tetraploid sexual species as follows: *D. conterminum* originated from hybridization between apogamous *D. takii* and sexual *Diplazium* sp. E; *D. virescens* originated from *D. takii* and sexual *Diplazium* sp. F; *Diplazium* sp. 1 originated from apogamous *D. dilatatum* and sexual *D.* sp. G; *Diplazium* sp. 2 originated from apogamous *D. doederleinii* and sexual *D.* sp. K; hypothesized diploid sexual *D.* sp. 4 originated from sexual *D. amamianum* and apogamous

D. sp. H; and tetraploid sexual *D. nipponicum* originated from hybridization between *D. amamianum* and diploid sexual *D.* sp. J. In addition, *Diplazium* sp. 1, *Diplazium* sp. 2, and *Diplazium* sp. 4 were very similar to *D. dilatatum*, *D. doederleinii*, and *D. hachijoense*. More *Callipteris* species (Wei et al. 2013), including members of the *D. hachijoense* complex, need to be collected from China and adjacent areas to further dissect such dynamics.

According to the relationships of diploid sexual species and triploid apogamous species, allelic inheritance patterns in the *D. hachijoense* complex were found to be consistent with the hybridization cycle hypothesis by Lin et al. (1992, 1995) and are similar to examples from other fern taxa. For example, Hori (2018c) reported that *Deparia okuboana* (Athyriaceae) potentially had a hybrid origin from the sexual diploid species *D. viridifrons* and the apogamous triploid species *D. unifurcata*, with the latter producing unreduced diploid sperm. In *Dryopteris* (Dryopteridaceae); several studies have reported that numerous triploid apogamous species share alleles with other triploid apogamous species and with diploid sexual species (Darnaedi et al. 1990, Lin et al. 1992, Hori et al. 2014, Hori et al. 2018b, Hori et al. 2018d). Ebihara et al. (2012) reported that tetraploid apogamous *Dryopteris shibipedis* had alleles in common with apogamous *D. pacifica* and the sexual tetraploid species *D. kinkiensis*.

Morita et al. (1990) reported a similar phenomenon in the angiosperm genus *Taraxacum* (*Asteraceae*), revealing that diploid sexual species endemic to Japan hybridize with triploid apogamous species introduced from Europe. The resulting *Taraxacum* are tetraploid or triploid apomicts. Therefore, the hybridization cycle is considered to be an important process that facilitates the production of triploid apogamous hybrids in plant reticulation complexes.

Conclusions

Continuous morphological variation in the *D. hachijoense* complex reflects a history of recurrent hybridization events among sexual and apomictic taxa, an observation in line with the hybridization cycle hypothesis suggested by Lin et al. (1992, 1995). The resulting hybrid apomict species is comprised of genomes derived from *D. amamianum*, *D. dilatatum*, *D. doederleinii*, and *D. takii*. More analysis of species distributed across China and adjacent areas is required in order to further comprehensively dissect the relationships between unknown lineages, undescribed species, and all members of the *D. hachijoense* complex.

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Appendix I

Voucher specimens examined in this study. Any allelic types of nuclear gene *AK1* that were identified by sequencing are in boldface. Otherwise, the allelic types of nuclear gene *AK1* were deduced from comparisons of band positions in SSCP gels. Data are in the order: Species name – locality, voucher (Herbarium), reproductive mode (sex: sexual, apo: apogamous), chromosome number, ploidy, haplotype of plastid *trnL-F*, allele of nuclear *AK1*. The haplotype of outgroups was only shown in Appendix 2 with accession numbers.

Members of the *D. hachijoense* complex

Diplazium amamianum **Tagawa** – JAPAN. **Kasgoshima pref.:** Amami city, Naze, Honchya-touge, 250m alt., May 7 2017, *K. Hatake 615* (KUMA), sex, 2*n* = 82, 2*x*, δ1, D1; ibid., Sumiyou village, Santaro-touge, 350m alt., May 26, 2018, *K. Hatake 985* (KUMA), sex, 2*n* = 82, 2*x*, δ1, **D1;** ibid., Naze, Ooaza-asato, May 6, 2017, *K. Hatake 609* (KUMA), sex, 2*n* = 82, 2*x*, δ4, **D1.**

Diplazium conterminum **Christ** – JAPAN. **Kagoshima pref:** Yakushima Is, Tabugawa, 200m alt., 130°36'55.92"N, 30°23'19.23"E, January 23 2019, *K. Hori 3158* (MBK), β1, **B2F**.

Diplazium dilatatum **Blume** – JAPAN. **Kagoshima pref.:** Amami city, Sumiyou village, Santaro-touge, 350m alt., September 7, 2017, *K. Hatake 705* (KUMA), sex, 2*n* = 82, 2*x*, α2, **A1;** ibid., Naze, May 24, 2018, *K. Hatake 974* (KUMA), sex, 2*n* = 82, 2*x*, α1, **A4;** ibid., Yakushima Is, Koseda, 70m alt., 130°39'24.54"N, 30°22'44.24"E, January 20 2019, *K. Hori 3082* (MBK), α1, **A6;** ibid.; *K. Hori 3083* (MBK), α1, **A1A2;** ibid., Hara, 80m alt., 130°34'19.72"N; 30°14'41.56"E, January 22 2019, *K. Hori 3125* (MBK), α1, **A6**. **Okinawa pref.:** Kunigami village, Mt. Yonahadake, *S. Serizawa 91648-1* (AICH), apo, α1, **A3A4;** Nago city, Genka, *S. Serizawa 91658-2* (AICH), α1, **A1A4.**

Diplazium doederleinii **(Luerss.) Makino** – JAPAN. **Kagoshima pref.:** Yakushima Is, Isso-river, 390m alt., 130°29'22.32"N; 30°25'13.09"E, January 23 2019, *K. Hori3173* (MBK), apo, γ2, **C.**

Diplazium hachijoense **Nakai** – JAPAN. **Chiba pref.:** Katori county, Tako-machi, Hayashi, October 25 2014, *K. Hori 1681* (MAK), δ3, **B2D2. Kagoshima pref.:** Tokunoshima Is, Mt. Inokawadake, 200m alt., September 12 2016, *K. Hatake 395* (KUMA), apo, 2*n* = 123, 3*x*, δ1, **B2D1**; ibid., January 13 2018, *K. Hatake 773* (KUMA), apo, 2n 123, 3x, δ1, B2D1; ibid., *January 13 2*018, *K. Hatake 776* (KUMA), apo, 2*n* = 123, 3*x*, δ2, B2D1. **Mie pref.:** Minamimuro county, Kihoucho, March 18 2018, *M. Takamiya 883, 893* (KUMA), apo, 2*n* = 123, 3*x*, δ3, **B2D2;** ibid., *M. Takamiya 919* (KUMA), apo, 2*n* = 123, 3*x*, δ5, **B2D3. Okinawa pref.:** Nago city, Genka, *S. Serizawa 91664-1* (AICH), δ1, B2D1. **Shizuoka pref.:** Shimoda city, Renndaiji-onsenn, 100m alt., 138°55'16.85"N; 34°41'55.23"E, May 22 2018, *K. Hatake 1010* (KUMA), apo, 2*n* = 123, 3*x*, δ5, B2D3. **Yamagu-**

chi pref.: Nagato city, Ichinoo, January 4 1996, *M. Takamiya 528* (KUMA), apo, 2*n* = 123, 3*x*, δ3, B2D2.

***Diplazium nipponicum* Tagawa** – JAPAN. **Kanagawa pref.:** Minamiashigara city, Kano, March 18 2018, *M. Takamiya 1004* (KUMA), sex, 2*n* = 164, 4*x*, ε, **D2J. Mie pref.:** Minamimuro county, Kiho-cho, 70m alt., 135°59'29.5"N; 33°45'55.2"E, July 6 2016, *K. Hori 2339* (MAK), ε, **D2J.**

***Diplazium okinawaense* Tagawa** – JAPAN. **Kagoshima pref.:** Yakushima Is, Isso-river, 200m alt., 130°28'21.08"N; 30°26'23.30"E, Jan 21 2019, *K. Hori3087* (MBK), apo, δ2, **A1B2.**

***Diplazium taiwanense* Tagawa** – JAPAN. **Kagoshima pref.:** Yakushima Is, Koseda, 70m alt., 130°39'24.54"N; 30°22'44.24"E, January 20 2019, *K. Hori 3080* (MBK), apo, α1, **A1A6B1;** ibid., 70m alt., 130°39'24.54"N; 30°22'44.24"E, January 20 2019, *K. Hori 3084* (MBK), β2, **A2B1**.

***Diplazium takii* Sa.Kurata** – JAPAN. **Mie pref.:** Minamimuro county, Kiho-cho, 70m alt., 135°59'29.5"N; 33°45'55.2"E, July 6 2016, *K. Hori 2343* (MAK), β1, **B2B3;** ibid.; *M. Takamiya 866* (KUMA), apo, 2*n* = 123, 3*x*, β1, **B2. Fukuoka pref.:** Kasuya county, Hisayama-machi, 140m alt., 130°32'2.27"N; 33°40'44.18"E, July 6 2016, *K. Hori 2924, 2958* (MBK), β1, **B2.**

***Diplazium virescens* Kunze** – JAPAN. **Mie pref.:** Minamimuro county, Kiho-cho, 70m alt., 135°59'29.5"N; 33°45'55.2"E, July 6 2016, *K. Hori 2341, 2342* (MAK), β1, **B2E. Kagoshima pref.:** Yakushima Is, Miyanoura river, 20m alt., 130°32'31.91"N; 30°24'48.36"E, Jan 21 2019, *K. Hori 3086* (MBK), β1, **B1B2E.**

***Diplazium* sp. 1** – JAPAN. **Okinawa pref.:** Nago city, Genka, *S. Serizawa 91663-1* (AICH), apo, α1, **A1A5G.**

***Diplazium* sp. 2** – JAPAN. **Okinawa pref.:** Kunigami village, Mt. Yonahadake, *S. Serizawa 91654-1, S. Serizawa 91654-2* (AICH), γ1, **CK.**

***Diplazium* sp. 3** – JAPAN. **Kagoshima pref.:** Kagoshima city, Chuzann-cho, Takinoshita-river, *M. Takamiya 1883* (KUMA), apo, 2*n* = 123, 3*x*, δ1, **CD2.**

***Diplazium* sp. 4** – JAPAN. **Mie pref.:** Minamimuro county, Kiho-cho, 70m alt., 135°59'29.5"N; 33°45'55.2"E, July 6 2016, *K. Hori 2338* (MAK), δ3, **D1H1;** ibid., March 18 2018, *M. Takamiya 929* (KUMA), apo, 2*n* = 123, 3*x*, δ1, D2H2. **Kumamoto pref.:** Amakusa city, June 14 2018, *K. Hatake 1030* (KUMA), apo, 2*n* = 123, 3*x*, δ1, **D2H2.**

Outgroups

***Diplazium chinense* (Baker) C.Chr.** – JAPAN. **Kochi pref.:** Agawa county, Niyodogawa-cho, Iwayagawa, 250m alt., 133°03'43.15"N; 33°32'22.21"E, June 16 2018, *K. Hori 3023* (MBK).

***Diplazium esculentum* (Retz.) Sw.** – JAPAN. **Kagoshima pref.:** Isa city, Oguchisogi, *M. Takamiya 1109* (MBK).

***Diplazium fauriei* Christ** – JAPAN. **Okinawa pref.:** Kunigami village, Mt. Yonahadake, *S. Serizawa 91656-1* (AICH).

***Diplazium mettenianum* (Miq.) C.Chr.** – JAPAN. **Mie pref.:** Minamimuro county, Kiho-cho70m alt., 135°59'29.5"N; 33°45'55.2"E, July 6 2016, *K. Hori 2336* (MAK).

***Diplazium wichurae* (Mett.) Diels** – JAPAN. **Kanagawa pref.:** Zushi city, Jinnmuji, 60m alt., 139°36'18.19"N, 35°18'14.71"E, July 6 2016, *K. Hori 1763* (MAK).

***Deparia japonica* (Thunb.) M.Kato** – JAPAN. **Kyoto pref.:** Sakyo-ku, Kibune, 300m alt., 135°45'50.79"N; 35°7'30.85"E, July 14 2018, *K. Hori 3031*(MBK).

***Deparia unifurcata* (Baker) M.Kato** – JAPAN. **Kochi pref.:** Agawa county, Niyodogawa-cho, Iwayagawa, 250m alt., 133°03'43.15"N; 33°32'22.21"E, June 16 2018, *K. Hori 3021* (MBK).

***Deparia viridifrons* (Makino) M.Kato** – JAPAN. **Kochi pref.:** Takaoka county, Ochi-cho, May 30 2018, *K. Hori 2971* (MBK).

***Athyrium crenulato-serrulatum* Makino** – JAPAN. **Tokyo pref.:** Ome city, Mt. Mitake, 880m alt., 139°08'32.09"N; 35°48'12.62"E, June 4 2018, *K. Hori 2979* (MBK).

***Athyrium decurrenti-alatum* (Hook.) Copel.** – JAPAN. **Tokyo pref.:** Hachioji city, Komagino, June 5 2018, *K. Hori 2986* (MBK).

Appendix 2

DNA data accession numbers of the obtained nucleotide sequences used for construction of molecular phylogenetic trees in this study. Data quoted from Hori (2018c) are marked by asterisks.

AK1

A1, LC468160; A2, LC468161; A3, LC468162; A4, LC468163; A5, LC468164; A6, LC468165; B1, LC468167; B2, LC468168; B3, LC468169; C, LC468166; D1, LC468172; D2, LC468173; D3, LC468174; E, LC468170; F, LC468171; G, LC468175; H1, LC468180; H2, LC468181; J, LC468176; K, LC468177; *Diplazium chinense*, LC468179, LC468182; *D. esculentum*, LC468186; *D. fauriei*, LC468183-LC468185; *D. mettenianum*, LC468178; *D. wichurae*, LC468187, LC468188; *Deparia japonica*, LC431726*; *D. unifurcata*, LC421961*; *D. viridifrons*, LC421960*; *Athyrium crenulatoserrulatum*, LC421516*; *A. decurrentialatum*, LC421512*.

trnL-F

α1, LC468195; α2, LC468196; β1, LC468199; β2, LC468200; γ1, LC468197; γ2, LC468198; δ1, LC468201; δ2, LC468202; δ3, LC468203; δ4, LC468204; δ5, LC468205; ε, LC468208; *Diplazium chinense*, LC468193; *D. esculentum*, LC468189; *D. fauriei*, LC468206; *D. mettenianum*, LC468207; *D. wichurae*, LC468190; *Deparia japonica*, LC468194; *D. viridifrons*, LC468191; *D. unifurcata*, LC468192.